Primer on Medical Genomics Part X: Gene Therapy

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Gene therapy is defined as any therapeutic procedure in which genes are intentionally introduced into human somatic cells. Both preclinical and clinical gene therapy research have been progressing rapidly during the past 15 years; gene therapy is now a highly promising new modality for the treatment of numerous human disorders. Since the first clinical test of gene therapy in 1989, more than 600 gene therapy protocols have been approved, and more than 3000 patients have received gene therapy. However, at the time of writing this article, no gene therapy products have been approved for clinical use. This article explains the potential clinical scope of gene therapy and the underlying pharmacological principles, describes some of the major gene transfer systems (or vectors) that are used to deliver genes to their target sites, and discusses the various strategies for controlling expression of therapeutic transgenes. Safety issues regarding clinical use of gene therapy are explored, and the most important technical challenges facing this field of research are highlighted. This review should serve as an introduction to the subject of gene therapy for clinician investigators, physicians and medical scientists in training, practicing clinicians, and other students of medicine.

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AAV = adeno-associated virus; ADA = adenosine deaminase; EPO = erythropoietin; FGF = fibroblast growth factor; HSC = hematopoietic stem cell; ICP = infected cell protein; IU = infectious unit; mRNA = messenger RNA; SCID = severe combined immunodeficiency; TK = thymidine kinase; VEGF = vascular endothelial growth factor

Gene therapy can be defined as any therapeutic procedure in which genes are intentionally introduced into human somatic cells.[1] However, a broader definition includes antisense therapy and related approaches in which short oligonucleotides are used to inhibit gene expression, as well as homologous recombination wherein nucleic acids are used to repair disease-causing mutations in the chromosomes of somatic cells. Of importance, this definition excludes genetic modification of the germline for therapeutic gain, which is currently banned in all countries. Indeed, great care is taken to avoid inadvertent germline gene transfer when gene therapy is administered.

In contrast to conventional small molecule drug therapies, which usually have a transient effect on their molecular targets, gene therapy usually results in a permanent change to the genetic constitution of the targeted somatic cells. Genes can be delivered directly to target cells in the body (in vivo gene therapy), or alternatively, the target cells can be explanted and genetically modified outside the body before they are reimplanted into the patient (ex vivo gene therapy) (Figure 1). Ex vivo gene therapy requires access to advanced laboratory facilities in which human cells or tissues can be processed in compliance with regulations of the Food and Drug Administration.

AIMS AND SCOPE OF GENE THERAPY

As aforementioned, gene therapy aims to change the genetic constitution of somatic cells by gene repair, gene suppression, or gene addition. Homologous recombination is the process by which gene defects can be repaired. The abnormal gene segment that contains a mutation, insertion, or deletion is excised and replaced; however, with current technology, the process is extremely inefficient such that only occasional cells are repaired correctly.[2] Gene suppression can be achieved through the use of short nucleic acid sequences that target specific messenger RNAs (mRNAs) in the cell. Ribozymes are more efficient than antisense oligonucleotides in this regard because they have catalytic activity and are able to cleave the target mRNA.[3] Similarly, inhibitory RNA is highly efficient because it recruits cellular enzyme complexes to degrade the targeted mRNA.[4] In gene addition therapy, normal copies of the gene are added to a cell without disrupting the expression of other genes. This can be achieved with reasonably high efficiency and provides the basis for most current gene therapy approaches.

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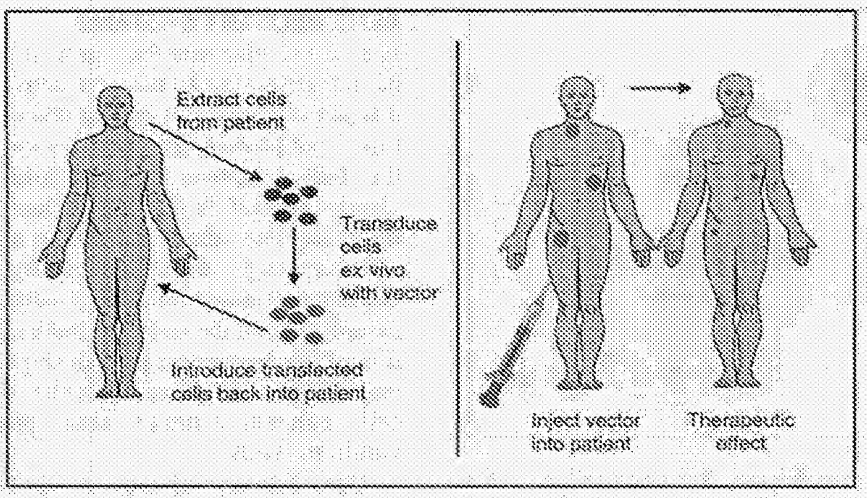

Figure 1. Ex vivo (left) and in vivo (right) gene delivery strategies (see text for details).

In principle, all human diseases are potentially amenable to gene therapy approaches. Four broad categories of gene therapy are recognized: compensation for gene defects, tissue engineering, cytotoxic or antiproliferative gene therapy, and immunostimulatory gene therapy.

Compensation for Gene Defects

Gene therapy has obvious appeal for the treatment of inherited single-gene disorders, particularly those for which current therapies are unsatisfactory or nonexistent. There are more than 4000 known single-gene disorders, and gene therapy for any one of these requires detailed knowledge of the genetic basis and pathogenesis of the disease. Certain mutations lead only to protein deficiency (eg, severe hemophilia) and are potentially amenable to treatment by adding normal copies of the damaged genes. Other mutations lead to production of a harmful mutant protein (eg, hemoglobin S and sickle cell anemia) and cannot be corrected unless the harmful protein can be suppressed. Therefore, the ideal approach to a single-gene disorder is full repair of the genetic defect, thereby ablating the normal protein and replacing it with its normal counterpart. However, as aforementioned, gene repair is technically challenging. An additional factor to consider in compensation for single-gene disorders is the reversibility of tissue pathology. For example, gene therapy for lysosomal storage disorders should be implemented before irreversible brain damage has occurred, and gene therapy for cystic fibrosis should be implemented before bronchiectasis has developed.

Tissue Engineering

Tissue engineering covers biomaterials science, cell and tissue culture methods, stem cell technology, and gene transfer technology. A central theme is the creation of genetically engineered cells or tissues with novel properties through the expression of intracellular proteins, membrane proteins, or secreted proteins having either short-range or long-range activities. Examples are as follows: (1) erythropoietin (EPO) gene transfer (eg, into muscle) or creation of EPO-secreting neo-organs to regulate red blood cell production in renal failure;[7] (2) expression of interleukin 1 antagonists in inflamed joints to suppress inflammation;[8] (3) expression of vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF) in ischemic tissues to promote angiogenesis;[9] (4) expression of nerve growth factor in neural stem cells implanted into the substantia nigra for treatment of Parkinson disease;[8] (5) expression of chimeric T-cell receptors in cytotoxic T cells to target them against cancer antigens;[9] and (6) expression of chemotherapy resistance genes in normal bone marrow progenitors to protect against chemotherapy-induced myelosuppression.[10]

Cytoreductive-Antiproliferative Gene Therapy

Cytoreductive-antiproliferative gene therapy has particular relevance not only in cancer but also in cardiovascular disease in which it can be used to prevent restenosis and vessel reocclusion by combating vascular smooth muscle proliferation after angioplasty. Genes used for cytoreduction include drug sensitivity genes, also known as suicide genes, which render cells sensitive to an otherwise nontoxic prodrug. The most widely used suicide gene is thymidine kinase (TK) from the herpes simplex virus, which phosphorylates the prodrug ganciclovir into a toxic drug termed *ganciclovir triphosphate*.[11] Under certain circumstances, death by apoptosis is preferred to necrotic cell

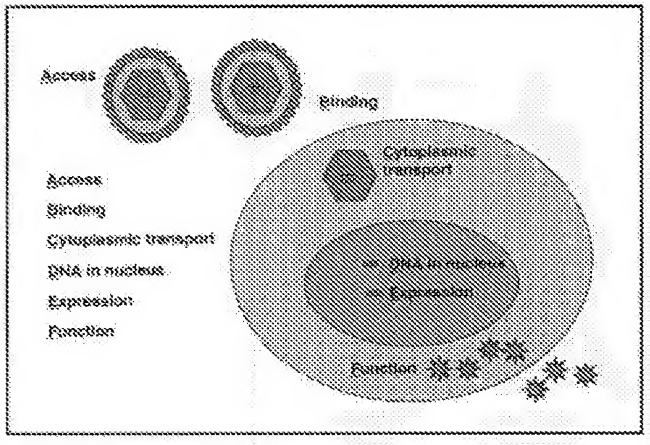

Figure 2. The ABCs of gene delivery. The target cell may be a stem cell, a fully differentiated cell, or a tumor cell. The vector must gain access to the target cell. Binding to the target cell leads to transfer of genetic material into the cytoplasm and transport into the nucleus where the DNA is transcribed (expressed). The next step is ribosomal translation, giving rise to a functional protein.

death and can be achieved through proapoptotic gene transfer using, for example, p53[12] or a dominant negative mutant of cyclin G.[13] Additional cytotoxic gene products being explored for cancer therapy include ribosomal toxins; fusogenic viral glycoproteins, which fuse tumor cells into large nonviable syncytia; and the thyroidal sodium iodide symporter, which traps radioactive iodine inside the transduced cells.[14,15]

Immunostimulatory Gene Therapy

Immunostimulatory gene therapy has particular relevance to the treatment of cancer and the prevention or treatment of infectious diseases. When the target antigen has been identified and cloned, the gene coding for the antigen (viral or tumor associated) can be delivered, for example, to muscle cells by using viral or nonviral vectors. Local production of the antigen is then sustained until the source is eliminated by the immune system. Alternatively, the gene can be introduced into antigen-presenting cells such as dendritic cells, which are then used as a cellular vaccine. If the antigen gene has not been cloned, then antigen-expressing cells can be genetically modified to create a cellular vaccine.[16,17] Genes coding for cytokines or other molecules that enhance the host immune response can be introduced into tumor cells, which are then used as a vaccine to provoke specific antitumor immunity. Genes coding for interleukin 2, interleukin 12, granulocyte-macrophage colony-stimulating factor, and the costimulatory molecule B7 have all proved to be effective in preclinical models.[18-20]

GENE DELIVERY SYSTEMS

A gene is a blueprint for a protein. Therefore, as a drug it has no activity until it has been delivered into the nucleus of a target cell, where it can be decoded and expressed as a functional protein. The key to successful gene therapy is the ability to deliver the therapeutic gene accurately, efficiently, and safely into the nucleus of the target cell and the ability thereafter to control its expression in the target cell. Key steps in the gene therapy process are access, binding and entry into target cells, transport across the cytoplasm into the nucleus, and transcription and translation of the therapeutic protein (Figure 2). The target cells may be stem cells, cancer cells, or fully differentiated cells, either in a tissue culture plate or at any location within the body.

Gene delivery vehicles, also known as vectors, are required for successful deployment of gene therapy, and their performance sets the boundaries for what can be attempted in human gene therapy.

Key elements of a typical vector include a nucleic acid component or expression cassette that comprises both the therapeutic gene and the regulatory elements that control gene expression and a vehicle whose purpose is to protect the nucleic acid from nucleases and to transport it to its destination in the nucleus of target cells (Figure 3). Key components of the vehicle include a surface element that mediates recognition of the target cell surface and elements mediating subsequent penetration into the correctly identified target cell. A typical gene therapy vector differs from a typical small drug in that it has multiple components, all of which can be engineered independently toward the goal of improved vector performance.

Nonviral Vectors

The 2 broad categories of gene delivery vehicle are nonviral and viral. Nonviral vectors are based on plasmid DNA that is grown in bacterial hosts such as *Escherichia coli*. Plasmids are circular DNA molecules that carry an antibiotic resistance marker gene and a bacterial origin of replication to facilitate their amplification in *E coli* (Figure 4). A mammalian expression cassette comprising a therapeutic gene with its associated regulatory elements can be inserted into the plasmid. As aforementioned, naked plasmid DNA is susceptible to degradation by nucleases and does not efficiently enter into mammalian cells. However, after intramuscular administration, plasmid DNA can enter into myocyte nuclei, leading to expression of the plasmid-encoded protein.[21] Viral, bacterial, and tumor antigens expressed in this way can provoke a protective or therapeutic immune response, often more efficiently than a corresponding protein-based vaccine.[22] This is termed *genetic vaccination*. An alternative approach to achieving in vivo

gene delivery to liver or muscle by using naked plasmid DNA is the so-called hydrodynamic approach, in which the DNA is injected into the circulation in a large volume of fluid.[23] Applying an electric current to the target site (electroporation) can further enhance the efficacy of gene transfer using naked plasmid DNA. However, for more efficient gene delivery to human tissue, plasmid DNA must be incorporated into a fully synthetic gene therapy vector, eg, using microprojectiles or cationic lipid-protein formations.[24]

With the gene gun approach, DNA is coated onto microscopic gold or tungsten particles (microprojectiles) that are accelerated toward mammalian cells or tissues using a device known as a *gene gun*. The microprojectiles penetrate the cytoplasmic and nuclear membranes of the target cells and deliver their plasmid DNA cargo to the cell nucleus with reasonable efficiency. This approach may be useful for gene transfer to explanted tumor cells or to easily accessible tissues such as skin where the target site is relatively well circumscribed.

Polyamines, polycationic lipids, or neutral polymers can be complexed with plasmid DNA, leading to charge neutralization (DNA is negatively charged), protection from nuclease digestion, and enhanced internalization into target cells.[25] Many such DNA nanoparticles have been developed for gene transfer applications, but compared to viral vectors nonviral gene transfer efficiencies remain low. New lipids and additional protein-peptide elements incorporated into DNA lipid formulations may enhance solubility, target cell specificity and efficiency of endosomal escape, or transport to the cell nucleus.[26,27]

In addition to the nonviral gene delivery systems aforementioned, DNA uptake can be enhanced by the application of an electric current to the target cells or tissues (electroporation)[28,29] or by its incorporation into microbubbles that are then burst in the vicinity of the target cell population by the application of high-frequency ultrasound (ultrasonoporation).[30,31]

Advantages of nonviral vectors include the high genome capacity of 30 to 40 kb and their lack of immunogenicity (it is difficult to induce an immune response against plasmid DNA). An additional advantage relative to viral vectors is the perception of a lower risk of harmful adverse effects (discussed subsequently). Important disadvantages of nonviral vectors include their relatively low transduction efficiencies and their transient expression profile, which typically peaks within 48 hours but is thereafter rapidly extinguished by 7 days. However, in some situations this may be an advantage, and it may be possible to prolong the expression profile by using plasmid DNA replicons incorporating mammalian origins of replication, eg, from the Epstein-Barr virus.[32]

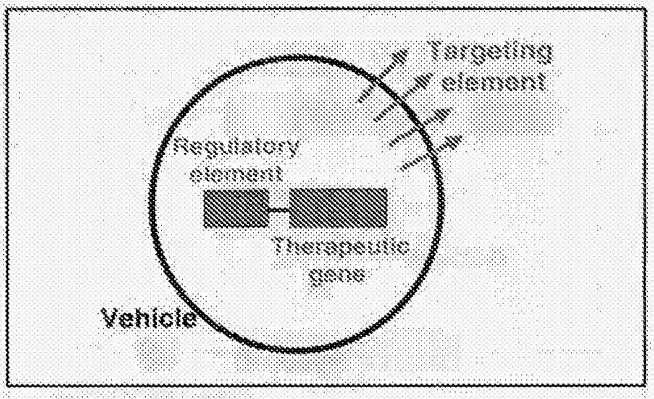

Figure 3. Schematic representation of a gene therapy vector (see text for details).

Viral Vectors

Many viruses efficiently deliver their nucleic acid genomes to mammalian cells as the initial critical step in their life cycle. Therefore, they have been perfected throughout millions of years of evolution for the task of gene delivery. The key to exploiting viruses as gene delivery vehicles is to introduce therapeutic genes into their genomes while concurrently removing the native viral genes that code for harmful viral proteins. The recombinant virus then functions purely as a vector that delivers the therapeutic gene to the nucleus of the target cell without causing cellular damage or subsequent virus propagation.

Viral vectors are generated by exploiting the packaging signal sequences that direct viral genomes into viral particles (Figure 5). A packaging signal sequence is a nucleic acid sequence contained within the viral genome that adopts a specific confirmation. Typically, the packaging signal sequence is recognized with high specificity by one of the structural proteins that participates in the assembly of the proteinaceous core of the virus. In a virally infected cell, the viral genome is copied and amplified, the viral genes are expressed, and the structural proteins are assembled to form new virus particles that interact with the progeny viral genomes guided by the all-important packing signal sequence to form fully infectious progeny virus particles that are released from the cell. To generate viral vectors, the packaging signal sequence is removed from the viral genome and appended to the therapeutic transgene. This packageable transgene is then introduced into a mammalian cell along with the viral genes, now lacking their packaging signal sequence such that the viral genes are expressed and new viral particles produced, but only the therapeutic transgene is packaged into the particles because it is now the only nucleic acid in the cell that carries the packaging signal sequence.

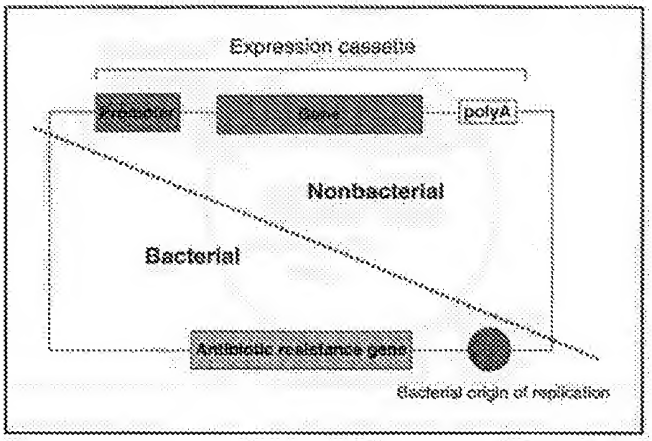

Figure 4. Schematic representation of an expression plasmid. The antibiotic resistance gene and bacterial origin of replication allow the plasmid to be grown in *Escherichia coli*. The expression cassette comprises a gene with associated regulatory elements to drive expression in mammalian cells.

Virtually any virus can be exploited as a gene delivery vehicle. However, at present, the most widely used viral vectors are derived from the following viruses: retrovirus (and lentivirus), adenovirus, adeno-associated virus (AAV), and herpes simplex virus.[33] Each viral vector has distinct characteristics that may make it more or less suitable for a particular gene therapy application. There is no perfect universal vector, and decisions about which vector to use for a particular application should be made on a case-by-case basis. A brief description of some of the major viral vector systems is provided subsequently.

Retroviral and Lentiviral Vectors.—Retroviral and lentiviral vectors are derived from C-type retroviruses such as murine leukemia virus[34] or from lentiviruses such as human immunodeficiency virus and feline immunodeficiency virus. The viral particles are roughly spherical, 80 to 110 nm in diameter, comprising an icosahedral protein core that contains 2 copies of the 7- to 11-kb single-stranded RNA viral genome plus 3 virally encoded enzymes: reverse transcriptase, protease, and integrase. The core is surrounded by a lipid envelope that carries the viral envelope glycoproteins responsible for virus attachment and entry. After attachment, the virus envelope fuses with the cell membrane, and the core moves toward the nucleus. The viral RNA is reverse transcribed to double-stranded DNA and transported into the nucleus where the integrase directs its insertion into the host chromosomal DNA at a random site. Viral genes are transcribed from the integrated (proviral) DNA. To make retroviral vector particles, 2 helper plasmids are expressed in a packaging cell, 1 coding for core proteins and viral enzymes and 1 for envelope glycoproteins. The packageable RNA that codes for the therapeutic protein is transcribed from a third plasmid, the vector plasmid. Murine leukemia virus–based retroviral vectors do not integrate or express in quiescent cells. Cell division is required for integration. In contrast, lentiviral vectors can integrate in quiescent cells. Integration is semirandom, using a different chromosomal site in each transduced cell with an overall preference for transcriptionally active target sites.[35] Expression of the transgene varies substantially from cell to cell according to the integration site. Random integration is associated with a risk of cell transformation (insertional mutagenesis) caused by disruption of a tumor suppressor gene or activation of a cellular oncogene.[36]

Retroviral and lentiviral vectors have a capacity of 8 kb and provide maximum titers up to 10^{10} IU/mL.[35] Because of integration, the transgene persists in the progeny of the originally infected cells.[38] Vector particles are immunogenic, but vector-transduced cells express no viral gene products and are therefore nonimmunogenic. The expression profile peaks within 72 hours and then gradually declines over weeks, months, or years because of transgene methylation, acetylation, provirus deletion, or death of the target cell.[39]

Adenovirus Vectors.—Adenovirus vectors are nonenveloped viruses with an 80- to 110-nm-diameter icosahedral protein shell that contains a 35- to 40-kb double-stranded DNA genome.[40] The fiber proteins appear on electron microscopy as prominent spikes at the 12 vertices of the icosahedron. Primary attachment to the target cell is through the fiber protein, and secondary attachment to cell-surface integrin receptors occurs through the penton base protein that anchors the fiber at the vertices of the icosahedron. After endocytosis, the virus disrupts the wall of the endosome and is released into the cytoplasm. The cytoplasmic virus migrates to the nuclear envelope and delivers the viral DNA to the nucleus. In wild-type adenovirus infection, early (nonstructural) viral genes are expressed initially, and the early proteins drive virus genome replication and late (structural) gene expression. To produce adenovirus vectors, early genes (eg, *E1*, *E4*) are deleted from the virus genome to disrupt the replication cycle, and therapeutic genes are inserted in their place. Vector particles are produced in cell lines that stably express the missing early gene products (eg, E1, E4) and can therefore support vector replication. In helper-dependent ("gutless") adenovirus vectors, all the viral coding sequences are removed. Production of helper-dependent vectors requires the addition of a replicating helper adenovirus that is later removed from the vector stock.

Adenovirus vectors have a capacity of approximately 8 kb for conventional vectors and 30 kb for helper-dependent

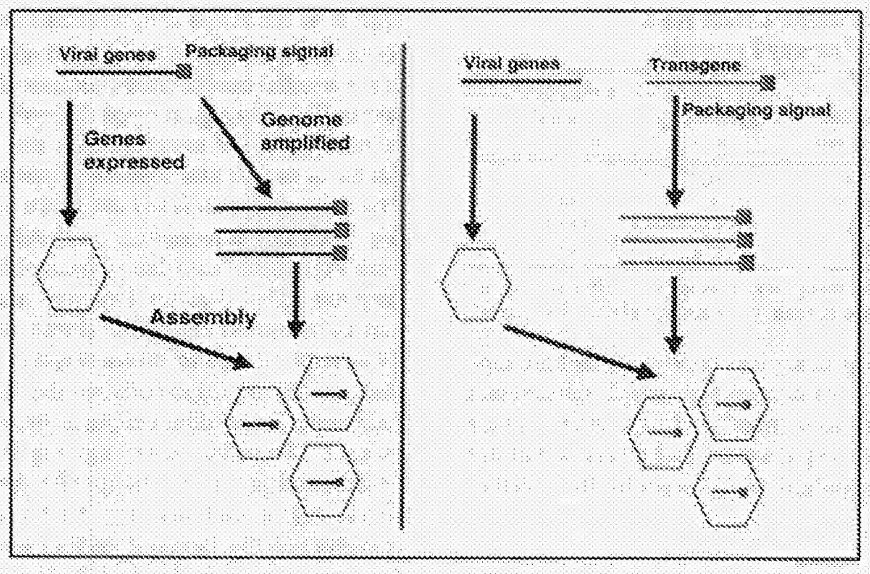

Figure 5. Schematic representation showing how the genomes of viruses and viral vectors are packaged into viral particles (see text for details).

vectors, and titers up to 10^{14} IU/mL are possible, allowing for high target cell transduction efficiencies. The vector genome persists in the cell as a linear, unintegrated episome and is therefore diluted by cell division. Adenovirus particles are immunogenic as are transduced cells in the setting of conventional adenovirus vectors because of low-level expression of viral structural genes. However, cells transduced with helper-dependent adenovirus vectors express no viral proteins and are not immunogenic. The adenoviral vector expression profile reaches an extremely high peak within the first 3 days and is then rapidly lost in the setting of conventional vectors because of immune-mediated destruction of transduced cells. However, with helper-dependent vectors, expression is maintained throughout weeks, months, or years because target cells are not subject to immune-mediated destruction.

AAV Vectors.—An AAV is an extremely small, nonenveloped icosahedral virus (18 to 26 nm in diameter); it carries a single-stranded DNA genome of approximately 5 kb with short, inverted terminal repeats required for genome replication and packaging. An apathogenic dependovirus, AAV replicates only in cells that are concurrently infected with a suitable helper virus (adenovirus or herpes virus). After virus attachment and translocation across the target cell membrane, the single-stranded DNA genome is transported to the cell nucleus where it is converted to double-stranded DNA, which is then transcribed by cellular polymerase. The AAV genome can persist in the cell nucleus, either as linear, unintegrated DNA or as integrated into the cellular chromosome.[39] To generate AAV vectors, the vector genome, comprising an expression cassette flanked by AAV-inverted terminal repeats, is introduced into mammalian packaging cells along with a plasmid coding for the AAV proteins and a second plasmid coding for necessary adenovirus helper functions.

Having a capacity of 5 kb, AAV vectors can be produced at titers up to 10^{12} particles per milliliter.[42] Adeno-associated virus genomes persist in the cell nucleus as episomal or integrated DNA. The particles are immunogenic, but transduced cells express no viral proteins. The transgene expression profile slowly increases during a period of weeks and then persists long-term with gradual decline.

Herpesvirus Vectors.—Herpes simplex virus is a fairly complex enveloped virus, 120 to 300 nm in diameter, that carries a double-stranded DNA genome of 152 kb. The icosahedral core, which houses the viral genome, is approximately 100 nm in diameter and is separated from the envelope by the tegument. Herpes simplex virus has a strong tropism for sensory neurons. Three waves of gene expression occur during the viral life cycle. Initially, the immediate early genes are expressed, and this leads to expression of the early genes, which in turn leads to expression of the late genes that generally code for viral structural proteins. Replication-defective herpesvirus vectors are constructed by removing critical immediate early genes such as infected cell protein (ICP)-4 and ICP-27 from the viral genome, which is then grown on complementing cells that stably express ICP-4 and ICP-27.[43-46] Herpesvirus vectors have a significantly higher capacity for foreign genetic

Table 1. **Vector Choice for a Gene Therapy Protocol**[a]

Vector	Transfer to progeny	Transduce nondividing cells	Efficiency of gene transfer
Nonviral	No	Yes	+
Retrovirus	Yes	No	++
Adenovirus	No	Yes	++++
Lentivirus	Yes	Yes	++

[a]Vector choice is guided by many factors; some of the key factors are shown here. Other issues include safety, accuracy, and size of transgene.

material than the vectors described previously and are rapidly gaining popularity for central and peripheral nervous system applications. Herpes simplex virus is able to enter into a latent state in sensory neurons, and this is one of the major reasons for its popularity as a vector for these cells.

Vector Choice

The choice of an appropriate vector system for a gene therapy protocol should be guided by consideration of the relevant properties of the different vector systems in relationship to the characteristics of the target cell population and the goals of the proposed application (Table 1). Thus, when introducing the β-globin gene into hematopoietic stem cells for correction of β-thalassemia, the real targets are the future erythroid progeny of the initially transduced cells. Therefore, efficient integration of the transgene into the host cell chromosome is highly desirable, an indication for the use of retroviral or lentiviral vectors. Alternatively, when a tyrosine hydroxylase or glial-derived neurotrophic factor gene is delivered to the striatum or substantia nigra for correction of Parkinson disease, the vector should efficiently and stably transduce quiescent neurons in the relevant parts of the brain without provoking an immune or inflammatory response and should lead to long-term, sustained production of the therapeutic protein. Both AAV and lentiviral vectors are attractive in this regard.[45-48] When the targets of therapy are cancer cells and the goal is to eliminate them, the highest priority is for a vector that can transduce the cells with an extremely high efficiency. In this setting, a strong immune/inflammatory response to the genetically modified cells is not contraindicated and may be desirable to increase the potency of the therapy. Therefore, conventional adenovirus vectors are appealing.

TARGETING DELIVERY AND REGULATING EXPRESSION

Transductional Targeting

There are 3 broad strategies whereby vectors can be targeted to accumulate at predetermined sites or selectively transduce a particular target cell population. In the first approach, the target cells are isolated and transduced in the tissue culture dish. In the second approach, regional delivery is used to ensure accumulation of vector at a particular site in the body, eg, aerosol delivery to airways,[49] a stereotactically guided injection into the brain,[50,51] or painting vector onto vascular structures during surgical exposure.[52] The third approach is to modify the vector (intrinsic targeting) such that it recognizes and transduces the target cells with high specificity but is incapable of transducing nontargeted cells.[53] For viral vectors, transductional targeting can be achieved by direct chemical modification of the virus coat, by use of bifunctional cross-linking molecules that provide a bridge between the vector and the cell surface target, or by direct engineering of the viral attachment proteins. Transductional targeting is an active area of research, and proof of principle has been established for all major vector systems. The first 2 clinical studies using transductionally targeted vectors were approved recently. One uses a retroviral vector displaying a collagen-binding peptide to enhance its retention in tumor blood vessels where collagen is highly exposed.[54] The other uses an adenoviral vector displaying an integrin-binding RGD peptide (the tripeptide, arginine-glycine-aspartic acid), which selectively enhances its ability to transduce ovarian carcinoma cells in the peritoneal cavity.[55]

In Vivo Barriers to Gene Delivery

In contrast to ex vivo gene therapy protocols in which a purified population of target cells is transduced in the culture dish, direct in vivo gene delivery is constrained by additional anatomical, biochemical, and physiological barriers. These include factors in body fluids such as antibodies and complement that can neutralize the vectors before they reach their target sites; the integrity of the endothelial lining of the blood vessels supplying target organs, which may prevent the vector from extravasating into the interstitial fluid where it can access its target cell population; and the distribution in the body of receptor sites for the vector, which may lead to massive sequestration of vector particles at sites that are not targets for gene transfer.

With respect to the immunological barriers to gene therapy, both humoral immunity to the vector or gene product and cell-mediated immunity to the genetically modified cells must be considered. Many patients have preexisting neutralizing antibodies against adenovirus, AAV, and herpesvirus vectors[56]; even if not present, these antibodies may develop after the first exposure to the vector. Preexisting immunity leads to variable neutralization of the first dose of vector or its gene product or elimination of the transduced cells. Maturation and amplification of the immune response after each exposure to therapy result in accelerated vector neutralization/transduced cell elimina-

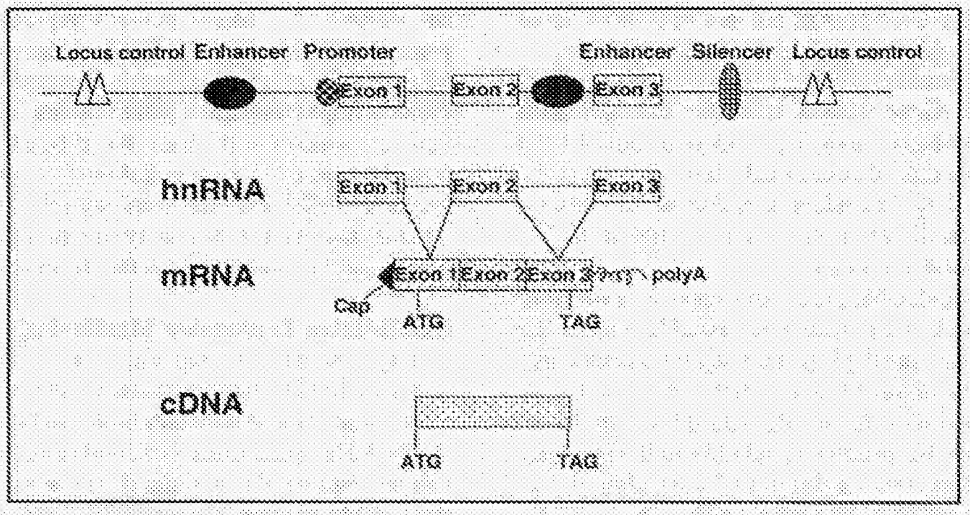

Figure 6. Schematic representation of a gene showing exons, introns, and regulatory elements. Heteronuclear RNA (hnRNA) is spliced in the nucleus to produce messenger RNA (mRNA). Complementary DNA (cDNA) refers to a DNA sequence comprising an exact copy of the mRNA sequences (see text for details).

tion with successive exposures. Thus, an ideal vector would not elicit an immune response in the treated patient.

Transcriptional Targeting

The narrowest definition of a gene is that it is a nucleic acid sequence that codes for a specific protein. In general, protein-coding sequences in a mammalian chromosome are divided into several exons separated from each other by long intronic sequences containing donor and acceptor sites for the cellular splicing machinery and are flanked by RNA processing signals that direct addition of a 5′ cap and 3′ polyadenylation signal and determine RNA stability. Transcriptional control elements, including the promoter, enhancers, silencers, and locus control elements, are also essential and integral components of the gene that function as landing pads for nuclear proteins (transcription factors) that regulate the level and timing of gene expression (Figure 6). The transcriptional promoter is located immediately upstream of the first exon. Transcriptional enhancer and silencer elements regulate the activity of the promoter element and may be located upstream or downstream of the gene or in one of the introns in either orientation, often a considerable distance from the promoter element. Locus control elements are typically found at a considerable distance from the coding sequences in a 5′ or 3′ direction and are the main determinants of chromatin conformation (open or closed) within a genetic locus.

Transcriptional control elements are portable and can be transferred from one gene to another, retaining their tissue specificity. Promoters and enhancers from housekeeping genes expressed in all tissues or from certain viruses (eg, cytomegalovirus) drive gene expression promiscuously in all transduced mammalian cells. Promoters and enhancers from genes expressed in a tissue-specific manner drive expression of foreign genes with the same tissue specificity.[57] Thus, the albumin promoter/enhancer is active only in hepatocytes,[58] tyrosinase promoter in melanocytes and melanoma cells,[59] immunoglobulin promoter/enhancer in B lymphocytes,[60] and β-globin promoter/enhancer in erythroblasts.[61,62]

β-Thalassemia major is a relatively common inherited disease characterized by deficient production of β-globin but continued high-level production of α-globin. Excess α-globin chains form α-4 tetramers that crystallize on the red blood cell membrane, leading to premature red blood cell destruction. Patients with this disease have severe anemia, are dependent on transfusions, and have a substantially shortened life expectancy. Bone marrow transplantation is curative; thus, for patients with no matched donor, the goal is reconstitution with genetically corrected autologous hematopoietic stem cells (HSCs). Lentiviral vectors expressing the β-globin gene under the control of a β-globin promoter/enhancer element drive erythroid-specific β-globin expression,[63] but the expression level varies considerably between cells, depending on the integration site. If globin locus control elements are introduced into the vector, the dependence on the integration site is eliminated, and gene expression levels in all transduced erythroid cells approach those seen with native globin genes.[63,64]

Pharmacological control of gene expression is desirable for certain gene therapy applications. Erythropoietin is

widely used for the treatment of anemia caused by renal failure or by malignancies such as multiple myeloma.[65,66] The protein is expensive and must be administered regularly by injection. Gene therapy has been explored as a potentially less expensive, more convenient means of EPO delivery. Administered subcutaneously at regular intervals, EPO leads to a dose-dependent increase in hematocrit. Therefore, dose titration is necessary to maintain the hematocrit within the desired range.

Several drug-regulatable gene expression systems have been developed. In the tetracycline-regulatable system, the therapeutic gene is controlled by a tetracycline-responsive promoter with low or zero basal activity. A second gene codes for a transcription factor that can drive expression from the tet-responsive promoter, but only in the presence of tetracycline. Therefore, addition of tetracycline drives transgene expression in a dose-dependent manner. Tetracycline-regulatable AAV vectors coding for EPO have been delivered to the muscles of laboratory mice and nonhuman primates. It has then been possible to control their hematocrit by treating the animals with different doses of tetracycline.[66,67]

PHARMACOLOGICAL PRINCIPLES OF GENE THERAPY

Pharmacokinetics is the study of the fate of a drug in the body. Because a therapeutic gene is inert, it must be converted to protein by the cells to which it has been delivered before it can exert any therapeutic effect. Therefore, it is important in gene therapy protocols to avoid focusing exclusively on the therapeutic gene and to study carefully the rates of production, accumulation, and elimination of the encoded therapeutic protein. Pharmacodynamics is the study of the way in which a drug mediates its characteristic actions (beneficial and harmful) in the body and includes the study of dose-response relationships. For gene therapy treatments, it is the protein product of the therapeutic gene that is of interest. The concentration of the therapeutic protein must be measured to determine the appropriate dose and dosing regimen for a gene therapy product.

Many of the proteins encoded by potentially therapeutic genes are cell associated and are not released into body fluids. Until recently, no satisfactory noninvasive methods existed for monitoring the accumulation of cell-associated proteins in the body. Thus, many clinical gene therapy studies failed to address the most basic issue of whether expression of the therapeutic gene was achieved. For example, recent cardiovascular gene therapy studies sought to promote angiogenesis in ischemic myocardium by delivery of proangiogenic molecules, VEGF and FGF-4.[68] Genes were delivered by direct intramyocardial injection or by coronary arterial perfusion using plasmid DNA or adenovirus vectors.[69,70] Many patients reported subjective improvement in their angina, but there was no direct evidence of gene expression, making it impossible to attribute the clinical improvement to gene therapy. Direct evidence of gene expression in the transduced hearts could not be obtained because (1) myocardial biopsy is dangerous and (2) because VEGF and FGF were not released into the bloodstream, and even if they were, the transgene-encoded proteins would be indistinguishable from native host proteins.

Noninvasive Expression Monitoring

Expression of a therapeutic gene can be monitored indirectly by linking its expression to that of a soluble marker polypeptide whose concentration can be measured in body fluids.[71] The therapeutic gene and soluble marker polypeptide are expressed concordantly (ie, at a constant ratio), and the soluble peptide is completely inert, meaning that it is nonimmunogenic and has no biological activity (Figure 7). Additional requirements for the marker peptide are that it should be secreted into the bloodstream, it should be absent in untreated individuals, it should have a known circulating half-life, and there should be a sensitive assay for accurate detection. Tumor markers such as carcinoembryonic antigen and the β-chain of human chorionic gonadotropin are good examples of suitable markers. Although soluble marker peptides can provide critical information on the profile of gene expression over time, they provide no information about the site of genetically modified cells.

For noninvasive mapping of the location/distribution of genetically modified cells, there has been considerable recent progress in the development of molecular imaging techniques in which a marker gene coexpressed with the therapeutic gene directs the production of a cell-associated marker protein that can be detected by radioisotopic imaging techniques. Examples include the thyroidal sodium iodide symporter, which concentrates radioiodine into the target cells in which it is expressed, allowing noninvasive detection by gamma camera imaging (iodine 123) or positron emission tomography (iodine 124).[72,73] Alternatively, herpes simplex virus TK expression can be detected by administration of a radioisotopically labeled substrate, which is phosphorylated by TK and thereby trapped inside the genetically modified target cell. Isotope trapping is then detected either by gamma camera imaging or by positron emission tomography.[73,74] Another approach is to use a marker gene coding for a nonimmunogenic cell-surface marker that can be detected by administration of a radioisotope-labeled peptide or monoclonal antibody.[75,76]

With the advent of these new noninvasive expression monitoring and molecular imaging strategies, it is possible to generate high-quality pharmacokinetic and pharmacodynamic data in the context of human gene therapy studies.

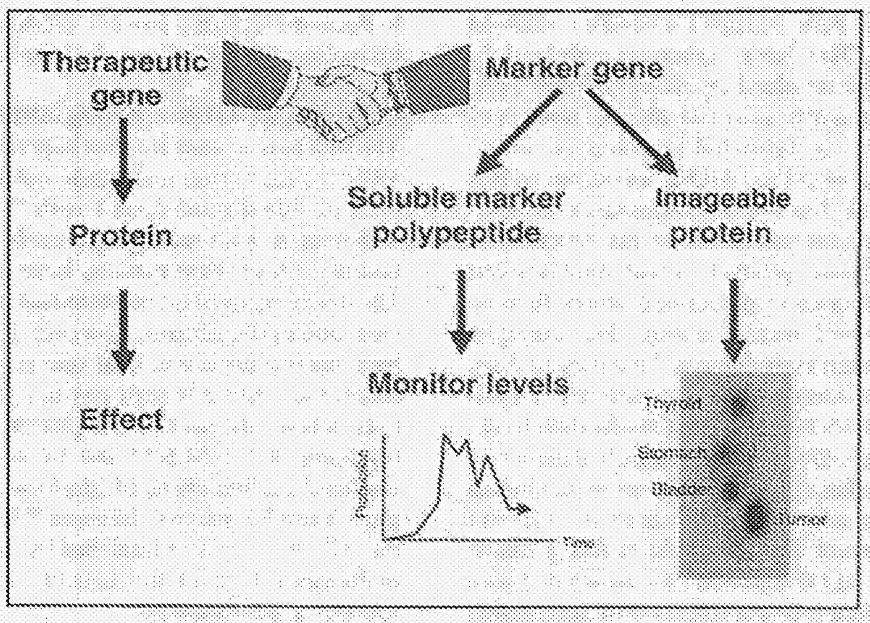

Figure 7. Noninvasive expression monitoring strategies. The therapeutic gene is linked to a marker gene such that it is expressed concomitantly. The marker peptide is then monitored by direct measurement or imaging, providing a surrogate measurement for therapeutic gene expression (see text for details).

This aids in addressing one of the major issues that has dampened enthusiasm for gene therapy in the pharmaceutical industry, how to define a dose of a gene therapy product. Ideally, a dose should be a quantity of vector that transduces a predetermined number of cells leading to production of a predetermined amount of protein, in turn leading to an expected therapeutic response in the patient with no toxicity. The major problem is that gene expression and protein production are highly variable between individuals given identical doses of vector particles by identical routes and between treatments in an individual given the identical dose on different occasions. Noninvasive expression monitoring strategies may allow routine titration of doses of gene therapy agents in individual patients.

SAFETY CONSIDERATIONS

Given that gene therapy is a new field of human therapeutic endeavor still in its infancy, data from human clinical studies are inadequate to address the numerous unanswered safety questions that remain. In theory, gene therapy is associated with risks to the patient, to the patient's future offspring, and to the general population. Toxicities to the patient may be caused either by the gene therapy vector or by its encoded gene product. For example, administration of the vector may result in anaphylaxis, inflammation, or infection, particularly if the stock is contaminated with a replication-competent version of the virus from which the gene therapy vector was derived. Direct liver toxicity was the cause of a widely publicized gene therapy fatality 3 years ago due to direct administration of an adenoviral vector into the hepatic artery of an 18-year-old man with ornithine carbamoyltransferase deficiency. This patient experienced rapid liver failure after infusion of a very high dose of the vector, but the pathogenesis was never fully elucidated.[7,8] Possible contributing factors include an inflammatory reaction to the virus particles, an inflammatory reaction to the virus-infected cells, and an inability to tolerate a liver insult due to the underlying ornithine carbamoyltransferase deficiency. To date, no human toxicities have occurred because of contamination of vector stocks with replication-competent viruses. Indeed, all gene therapy regulatory agencies have taken substantial steps to ensure that manufacture and testing of gene therapy products are conducted in such a way as to reduce this risk substantially.

Certain gene therapy approaches are associated with a finite risk of cancer due to either insertional mutagenesis or expression of an oncogenic protein. Insertional mutagenesis is a particular risk associated with the use of retroviral or lentiviral vectors that integrate randomly into the host cell chromosome and can therefore disrupt a tumor suppressor gene or activate expression of an oncogene. Indeed, recently, 2 of 11 children developed T-cell malignancies

after they had received gene therapy for severe combined immunodeficiency (SCID) due to common γ-chain deficiency.[79-81] The protocol involved ex vivo retroviral transduction and subsequent reinfusion of autologous bone marrow; in both situations, the retroviral insertion site in the malignant clone was adjacent to LMO-2, an oncogene that is known to be implicated in T-cell malignancies. In addition to insertional mutagenesis, there are theoretical concerns that certain transgene products might stimulate cell proliferation in an autocrine or paracrine fashion, thereby leading ultimately to cell transformation. For example, when cytokine genes such as interleukin 2 are used to drive the activation and proliferation of tumor-reactive T lymphocytes, there is an associated risk that inadvertent transduction of the T cells could result in uncontrolled autocrine growth. However, this has not been observed in preclinical or clinical studies in which a wide range of different cytokines have been used. Protein products that promote angiogenesis, such as VEGF and FGF, are associated with a theoretical risk of tumor growth promotion by stimulating the development of tumor neovessels. Patients enrolled in proangiogenic gene therapy protocols are being monitored closely for the appearance of new malignancies.

The theoretical concern of inadvertent germline gene transfer is addressed routinely in the preclinical studies conducted in support of new gene therapy protocols. Although the transgene may often be detected by polymerase chain reaction in gonadal tissues of experimental animals, there has been no documented case in which this has resulted in genetic modification of the germline. In fact, efforts to use gene transfer vectors with the intent of modifying the germline have had limited success, achievable only by isolation and direct inoculation of vector into the germ cells. To date, no human clinical trials have shown evidence of germline transduction by gene therapy vectors. However, in a recent study in which AAV vectors coding for human factor IX were administered to patients with severe hemophilia B, semen samples from one of the patients tested strongly positive for the vector sequences by polymerase chain reaction.[82] In contrast to germ cells, embryonic tissue can be transduced by gene therapy vectors, and gene therapy should generally not be used in pregnant patients.

Risks to patients must be evaluated when replicating viral vectors are used for therapy and when a viral vector stock may be contaminated with replication-competent viruses generated by recombination between vector and helper constructs during the manufacturing process. To avoid the possibility of introducing a transmissible viral pathogen into the patient or creating a new viral pathogen, human gene therapy studies are regulated stringently, and the Food and Drug Administration pays particular attention to the manufacturing process, product characterization, and toxicology testing of new viral vectors.[83]

CLINICAL STATUS AND PROSPECTS

The first human gene transfer experiment was performed in 1989; a patient with malignant melanoma received genetically modified autologous T cells.[84] Since then, more than 600 human gene therapy protocols have been approved, and more than 3000 patients have received gene therapy. The disorders most often treated are cancer, vascular occlusion, and cystic fibrosis; however, a large number of trials have been approved to treat rare genetic diseases. In most cases, therapeutic benefit has not yet been shown. However, a few successes have occurred, most notably in gene replacement for SCID[85] and in suicide gene transfer to facilitate the treatment of graft-vs-host disease occurring after donor lymphocyte infusion.[86] Nonetheless, the first of these 2 successes was tarnished by the occurrence of T-cell malignancy in 2 of the patients who had favorable responses to gene therapy.

Somatic gene therapy is appealing for SCID because the target cells for gene transfer (HSCs or T cells) are amenable to ex vivo culture and genetic modification, corrected cells have a significant survival advantage in vivo, and even a low efficiency of gene transfer should be sufficient for partial phenotypic correction. In addition, the risks of gene therapy are justified for SCID because of the poor prognosis of patients who do not have the option of a matched sibling HSC transplant. The first group of SCID patients treated with gene therapy were those with adenosine deaminase (ADA) deficiency. The first 2 ADA-deficient patients treated with infusions of their own genetically corrected lymphocytes had a favorable response to the therapy, and promising results were obtained in subsequent studies in which the *ADA* gene was transferred to autologous HSCs.[85] However, the most notable success has been in the treatment of X-linked SCID due to mutation in the gene coding for the common γ-chain.[79,80] $CD34^+$ bone marrow cells from boys with X-linked SCID were transduced ex vivo with use of a defective retroviral vector, and the γ-chain–expressing cells were reinfused without myeloablation. Of the first 5 patients to be treated with this protocol, 4 had a favorable response to therapy with correction of their immunodeficiency and resumption of a normal life. After the publication of these encouraging clinical responses, 6 additional patients were treated; however, 2 of the earlier treated patients recently developed clonal T-cell malignancies, both characterized by retroviral insertion adjacent to the *LMO-2* gene. Both patients are currently receiving therapy for their T-cell malignancies, and the other treated patients are being closely monitored for the development of clonal T-cell expansions.[81]

Thymidine kinase gene transfer was used successfully in allogeneic bone marrow transplant recipients who had recurrent malignancies.[86] Eight patients who experienced relapse after their allogeneic procedure were treated with donor T cells that were first transduced with a *TK* suicide gene using a retroviral vector. Five of the patients had a favorable response to therapy, and 3 subsequently developed graft-vs-host disease, at which point ganciclovir was administered to eliminate TK-positive donor lymphocytes. All 3 patients had a favorable response to this intervention. With the increasing popularity of miniallogeneic transplants and donor lymphocyte infusions, this suicide gene transfer strategy is of considerable importance.

Many other human gene therapy studies have generated encouraging clinical anecdotes. However, to date, no conclusive phase 3 studies have shown efficacy of gene therapy products, and the poor performance of currently available vectors continues to be a major limiting factor in gene therapy. Current research is focused on developing high-titer, targetable, regulatable, injectable vector systems that will allow highly efficient and accurate transfer of genes to target tissues in vivo. To accommodate the steady stream of new, improved vectors, the process of gene therapy research must incorporate iterative cycles of preclinical development and clinical testing (Figure 8).

We thank Maureen A. Craft for secretarial assistance.

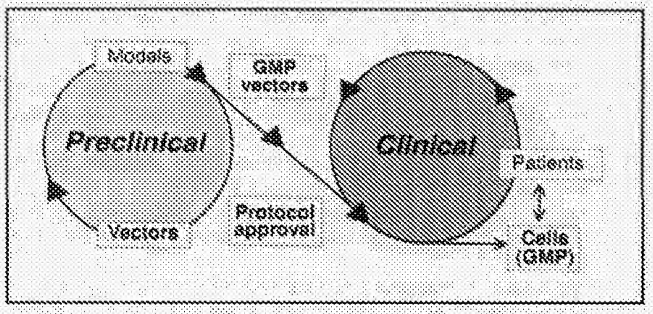

Figure 8. Gene therapy process. In the preclinical cycle, vectors are designed, constructed, tested, and perfected until they show activity that warrants clinical testing. Manufacturing, toxicology testing, and protocol development activities then lead to the clinical testing cycle in which patients are treated, outcomes are evaluated, and the clinical strategy evolves. Clinical outcomes may feed back into the preclinical cycle, guiding the construction and eventual clinical evaluation of new, improved vectors. GMP = good manufacturing practices.

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